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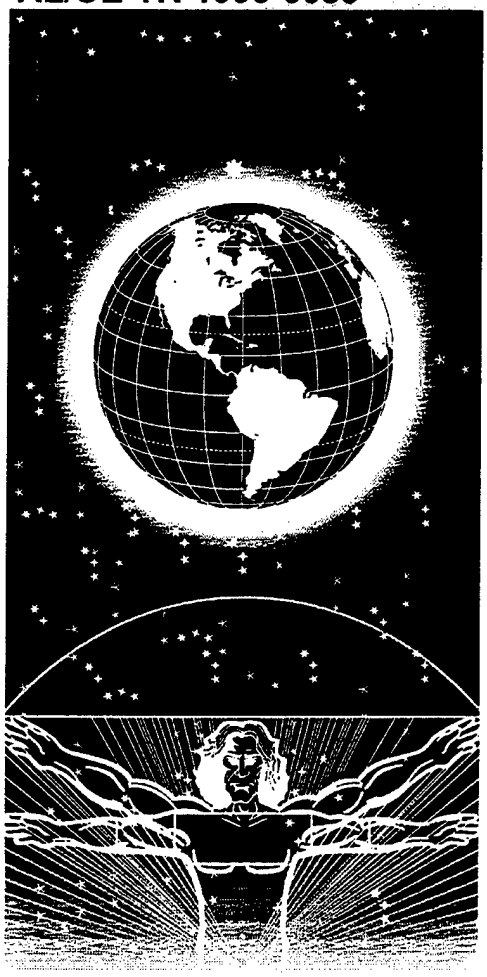

UNITED STATES AIR FORCE ARMSTRONG LABORATORY

QUANTITATION OF FREE RADICALS IN B6C3F1 MOUSE LIVER SLICES ON EXPOSURE TO FOUR CHEMICAL CARCINOGENS: AN EPR-SPIN TRAPPING STUDY

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TECHNICAL REVIEW AND APPROVAL

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

STEPHEN R. CHANNEL, Maj, USAF, BSC
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Free radicals generated in mouse liver slices following exposure to four chemical carcinogens: tert-butyl hydroperoxide (TBOOH), bromotrichloromethane (BrCCI3), carbon tetrachloride (CCI4) and trichloroethylene (TCE), were quantitated using electron paramagnetic resonance spectroscopy (EPR)/spin trapping techniques. Precision cut liver slices (n=128) were prepared from B6C3F1 mice and incubated in Waymouth's media supplemented with 10% fetal bovine serum and 10 mM N-tert-butyl-*a*-phenyl nitrone (PBN). Liver slices were exposed to control media or media with 1 mM TBOOH, BrCCI3, CCI4, or TCE. After 5 or 60 min. the slices and media were homogenized, frozen in liquid nitrogen and lyophilized. Radicals trapped by PBN were quantitated using an EMS 104 EPR analyzer (Bruker Instruments, MA). Standards were prepared by homogenizing the liver slices with known quantities of the spin label 2,2,5,5,-tetramethyl-1-pyrolidinyl oxy-3-carboxamide (3-CAR). EPR spectra were compared quantitatively and qualitatively. Two factorial analysis and analysis of variance showed there was significant differences in the radicals detected with time and chemical treatment ($P<0.002$). The EPR spectra of the lyophilized liver homogenate suggest there is a difference in the radical species trapped if slices are incubated in media containing 1 mM TBOOH, BrCCI3, CCI4, or TCE when compared to control media. The interaction of both time and concentration should be addressed in any studies designed to quantitate free radical-induced liver damage.

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PREFACE

This is one of a series of technical reports generated from the experimental laboratory programs conducted in the Electron Paramagnetic Resonance laboratory for the Pharmacodynamic Group, Armstrong Laboratory, Toxicology Division. The research described in this report began in December 1994 and was completed in September 1995. The data reported was presented at the Society of Toxicology Meeting in Annahein, CA 9-15 March, 1996. Liver samples were obtained from CPT Clay Miller, USA under a program sponsored by AFOSR Environmental Initiative Program Work Unit #2312A202 managed by Dr Frazier, S&T. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

The authors gratefully acknowledge the following : SRA S Trivunovic and SSgt G. Miller for liver viability determinations.

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ABBREVIATIONS

$BrCCl_3$	Bromotrichloromethane
3-CAR	2,2,5,5- Tetramethyl-1-pyrrolidinyloxyl-3-carboxyamide
CCl_4	Carbon tetrachloride
d	Day
EM	Electron microscopy
EPR	Electron Paramagnetic Resonance spectrometer
g	Gram
h	Hour
kg	Kilogram
L	Liter
mg	Milligram
ml	Milliliter
mm	millimeter
N	number
p	Probability
PBN	N-tert-butyl-α-nitrone
SD	Standard deviation
SEM	Standard error of the mean
TCE	Trichloroethylene
TBOOH	Tert-butyl hydroperoxide

INTRODUCTION

Increased lipid peroxidation occurs in liver after exposure to tert-butyl hydroperoxide (TBOOH), bromotrichloromethane ($BrCCl_3$), carbon tetrachloride (CCl_4), or trichloroethylene (TCE), Fraga et al. (1989), Kennedy et al. (1989), McCay et al (1989), von Ruecker et al. (1989), and Castilho et al. (1995). These chemicals were therefore chosen to see if the lipid peroxidation measured in liver is *caused* by free radicals induced by the parent compound or its metabolites, or whether the lipid peroxidation described is an *effect* of the cellular damage of the chemical. The hypothesis tested was *free radicals released in tissues by these four chemicals are responsible for and quantitatively linked with the lipid peroxidation and tissue damage*. To reduce sample variability a technique where liver of several B6C3F1 mice are pooled and slices of equal sizes prepared was used (Fraga et al., 1989). To see if the lipid peroxidation measured is caused by free radicals induced by the parent chemical or its metabolites, these four chemicals were used at concentrations which did not alter liver viability. Lipid peroxidation was assessed by measurement of TBARS (Fraga et al., 1988). It was necessary to develop a method to measure free radicals which would support the TBARS data using another analytical technique. For this project our aim was *to develop a method which would provide reliable quantitation of the free radicals generated by the known concentrations of parent chemical for correlation with the rate of lipid peroxidation.* The only definitive technique to detect free radicals is electron paramagnetic resonance (EPR), Buettner (1987). Preliminary studies, using liver slices with TCE, suggested lipid peroxidation is caused by TCE-induced radicals in a dose dependent manner, Steel-Goodwin et al.

(1994). Except for these preliminary studies, Steel-Goodwin et al., (1994), quantitation of biologically induced free radicals using EPR technology has been limited to the determinations of whether biological tissue has been irradiated or not. EPR was used in this study to determine if total free radical values were altered by chemical exposure or not. As a standard for these experiments the spin label 2,2,5,5,-tetramethyl-1-pyrrolidinyloxyl-3-carboxyamide (3-CAR) was used to provide a quantitative reference. The total radicals generated by the four carcinogens were determined at two time points and compared to samples of liver slices without chemical treatment. All samples were read at the same time, and the experiment was repeated four times, to determine the variation from one EPR run to another.

MATERIALS AND METHODS

Chemicals

Tert-butyl hydroperoxide (TBOOH), bromotrichloromethane ($BrCCl_3$), carbon tetrachloride (CCl_4), trichloroethylene (TCE) N-tert-butyl-α-nitrone (PBN) and 2,2,5,5,-tetramethyl-1-pyrrolidinyloxyl-3-carboxyamide (3-CAR) and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemical Co. or Sigma Chemical Co., St. Louis MO.

Sample Preparation

Male B6C3F1 mice 12 weeks old weighing 25-30 g were purchased from Charles River, Portage Laboratories, MI. All mice were euthanised by carbon dioxide asphyxiation and necropsied. The liver was immediately excised, cored using a stainless steel bore tool and cut into slices of equal thickness and size (Smith et al., 1987, Azari et al., 1990). Slices were pre-incubated in Waymouth's media for 2 h. The slices were then incubated in the Waymouth's media supplemented with 10 mM PBN with or without 1 mM TBOOH, $BrCCl_3$, CCl_4, or TCE. Each sample consisted of two slices. Slices were incubated for 5 min. or 60 min., homogenized, and 200 ul was removed for TBARS analysis. The remainder was immediately frozen in liquid N_2 and lyophilized in the dark. The study was performed on liver judged to be viable based on measurements of intracellular potassium content (AVL982-5 Electrolyte Analyzer, Roswell GA) and leakage of the liver enzymes lactate dehydrogenase (LDH), aspartamine transaminase (AST) and alanine transaminase (ALT). LDH was measured using the ACA chemical

analyzer (Dupont Co., Wilmington, DE) and AST and ALT were measured using the Kodak Ektachem Clinical Chemical Assay (Eastman Co., Rochester, NY).

General Experimental Design

The total radicals in the lyophilized samples were measured using a Bruker EMS 104 EPR analyzer. The machine parameters for the EPR analyzer were: microwave power, 25 mW; sweep width, 100 G; modulation amplitude, 4.02 G; sweep time, 10.49 s; filter time constant, 20.48 ms; receiver gain, 60. The spectra were measured by peak height directly from the EMS 104 EPR analyzer and by double integration with normalization for receiver gain using the EPR program (Bruker, Billerica, MS).

Statistics

All results were normalized by liver dry weight and analyzed by one way and two-factorial analysis of variance using the statistics package Design Ease®. Standard deviations and regression correlation were performed using Sigma Plot®.

RESULTS & DISCUSSION

Figure 1A shows the typical EPR spectrum obtained with homogenate of liver slices before and after lyophilization.

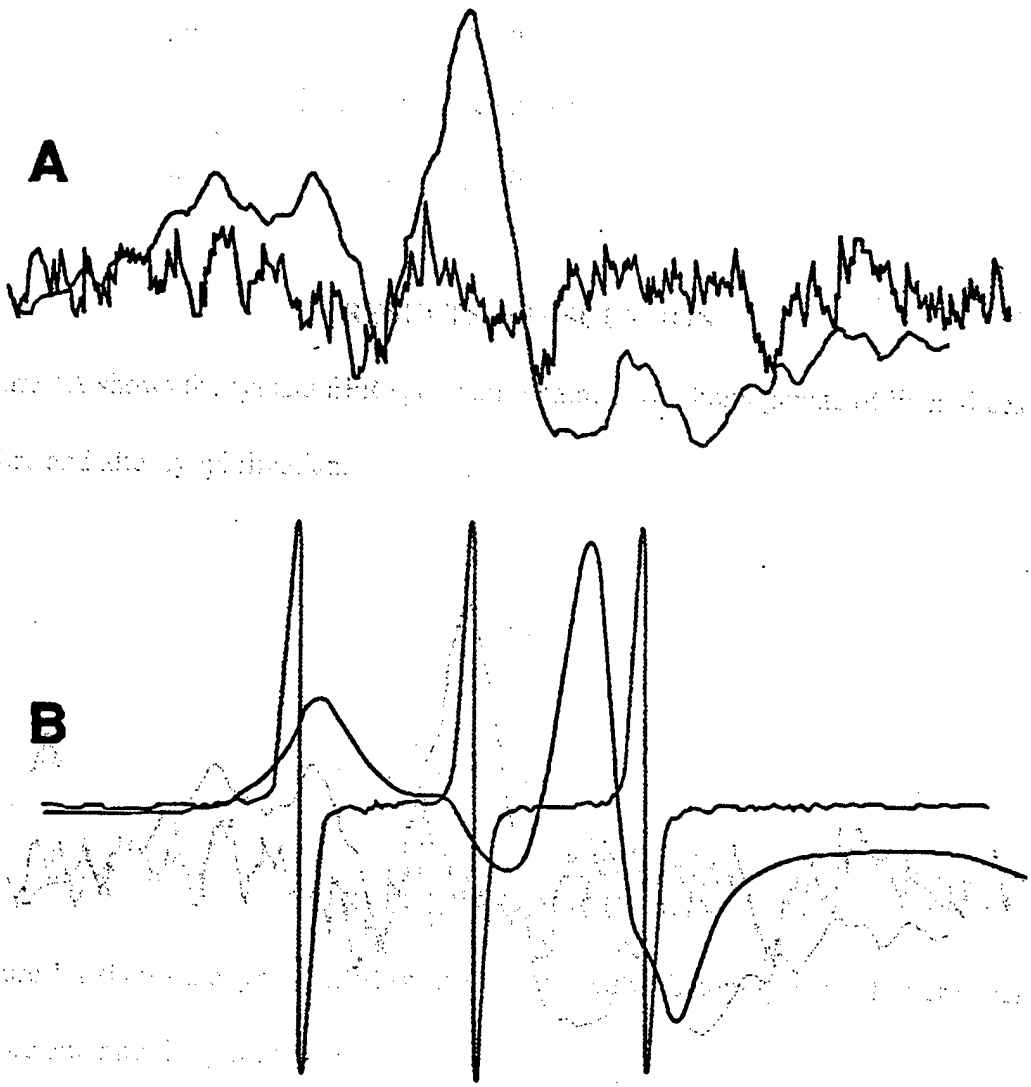

Figure 1. ***Typical EPR spectra of homogenates of (A) Control liver slices (B) 3-CAR standards, before and after lyophilization***

Radicals were not detectable in the homogenate and lipid peroxidation was also not detectable (Miller et al., unpublished data and Channel et al., unpublished data). To detect radicals, the samples were lyophilized. Figure 1B is the typical EPR spectrum before and after lyophilization of 2,2,5,5,-tetramethyl-1-pyrrolidinyloxyl-3-carboxyamide (3-CAR). 3-CAR is a stable radical or spin label which we used as the standard to quantitate radicals in these experiments. Known amounts of this spin label was added to liver slices in Waymouth's media producing the typical lyophilized spectrum seen in Figure 1B.

Lyophilized spectra formed in liver samples exposed to 1 mM TBOOH, $BrCCl_3$, CCl_4, or TCE for 60 min. are shown in Figure 2.

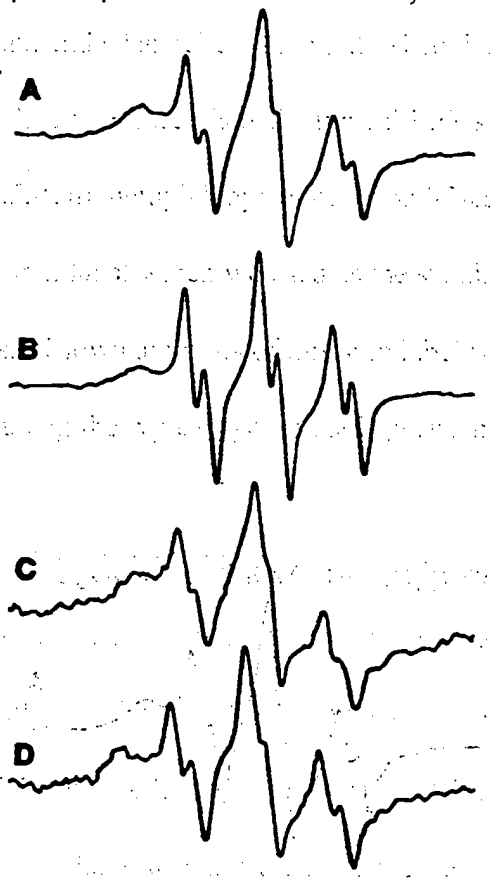

Figure 2. ***Typical EPR spectra of lyophilized liver slice homogenates exposed to 1 mM TBOOH, $BrCCl_3$, CCl_4, or TCE.***

The PBN-spectral adducts provide little information about the parent radicals. The radicals generated by these chemicals have been described elsewhere, Buettner (1987). This study was quantitative not qualitative. Our aim was to develop a method which would permit routine analysis of large numbers of samples using 3-CAR as a standard. The accuracy, sensitivity, and precision of the method was assessed. The performance of the machine to provide a reproducible result over the period of the experiments was determined using the pitch calibrator. The EPR machine was internally set to read pitch at 1000 arbitrary units (a.u.), Figure 3. The value for pitch in these experiments was 998.6 ± 1.3 (mean ± SD, n=40). While the computer of the EPR defaulted to measure peak height, double integration of the spectra of the samples was also performed.

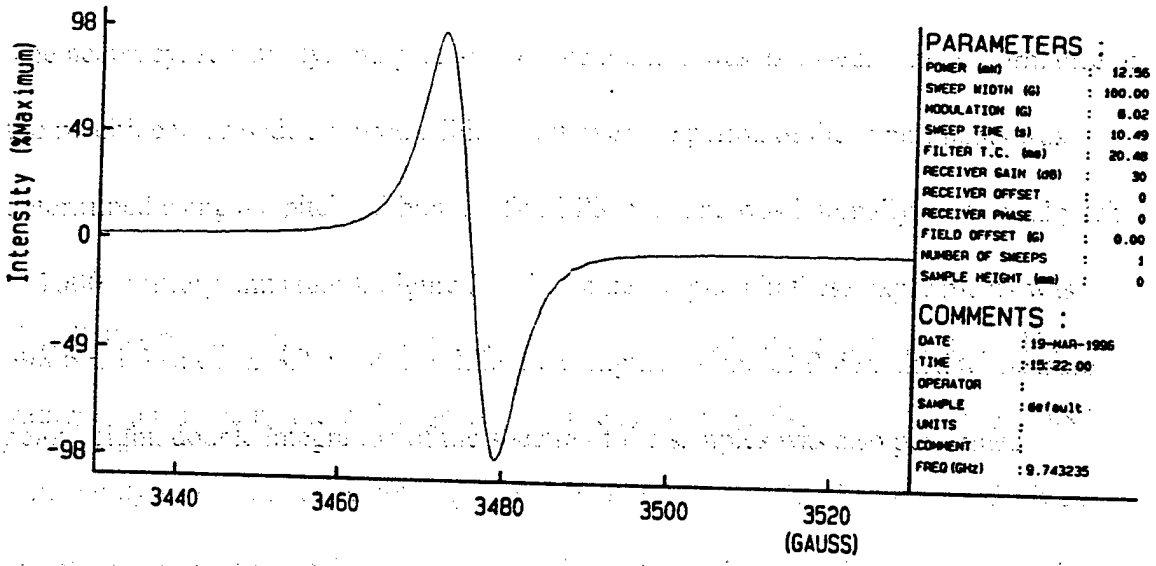

Figure 3 ***Spectrum of pitch***

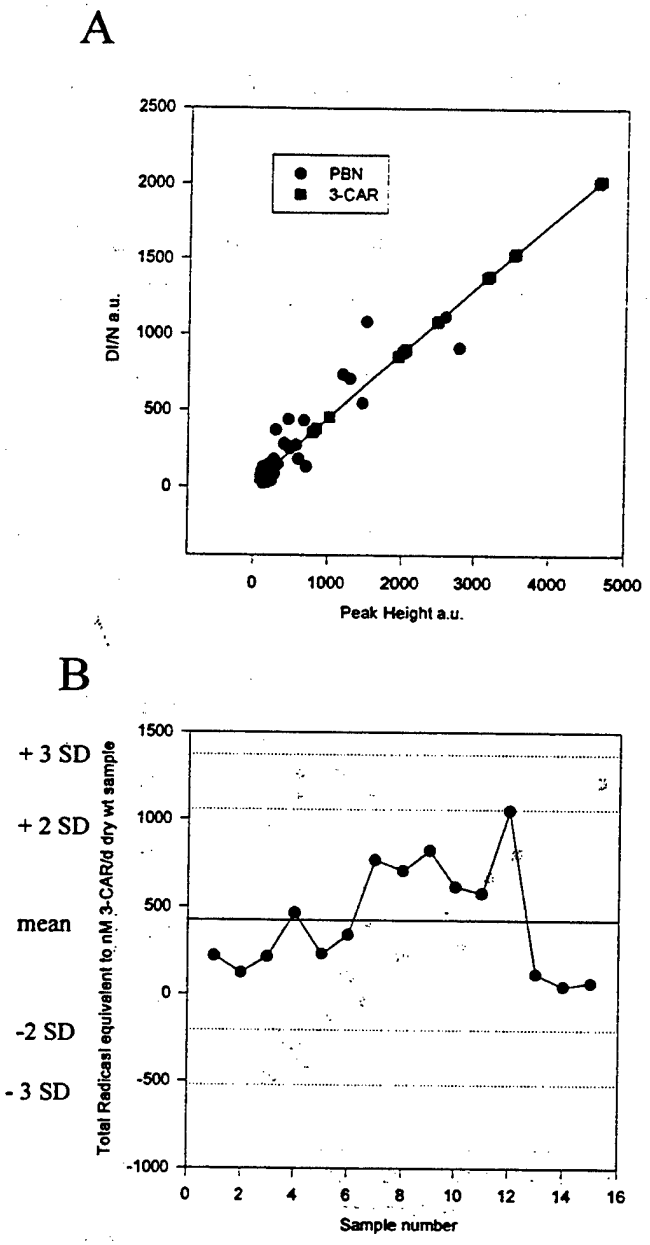

Figure 4 (A) ***Correlation of peak height measurement with double integrations of EPR spectra. (B) Variation of radical measurements in control liver slices over a 30-day study period.***

Figure 4A shows the correlation between the peak height and the DI/N of the samples in the spin trap PBN ($y = 0.43 x + 15.71.$, $r^2 = 0.84.$, $P< 0.001$). Corresponding values of the 3-CAR standards are also shown. Subsequent studies performed by Steel-Goodwin and Hutchens (1995) suggest that the trapping efficiency of PBN is only about 33% of the total radicals in the samples. Steel-Goodwin and Carmichael (1995) performed radiation experiments and using bond energies calculated the trapping efficiency for PBN for the TCE radical to be approximately 24%. PBN can be metabolized by liver enzymes to become EPR silent. Thus, as far as possible all samples for comparison were performed at the same time, so that the data is generated from the same population. Variation in control samples from different slice preparations over a 30 day period are shown in Figure 4B. Untreated samples gave values of 425.2 ± 315.6 (mean $\pm$ SD, n= 16). This variation strongly suggests liver preparations were not the same from one slice run to another. There was also variation in TBARS results with different media preparers. EPR is a non-destructive technique which detects the radicals spinning in the sample while it is exposed to a magnetic field. Thus, to show that the EPR analysis technique was reproducible, samples from control and treated liver slices were measured in four separate EPR runs.

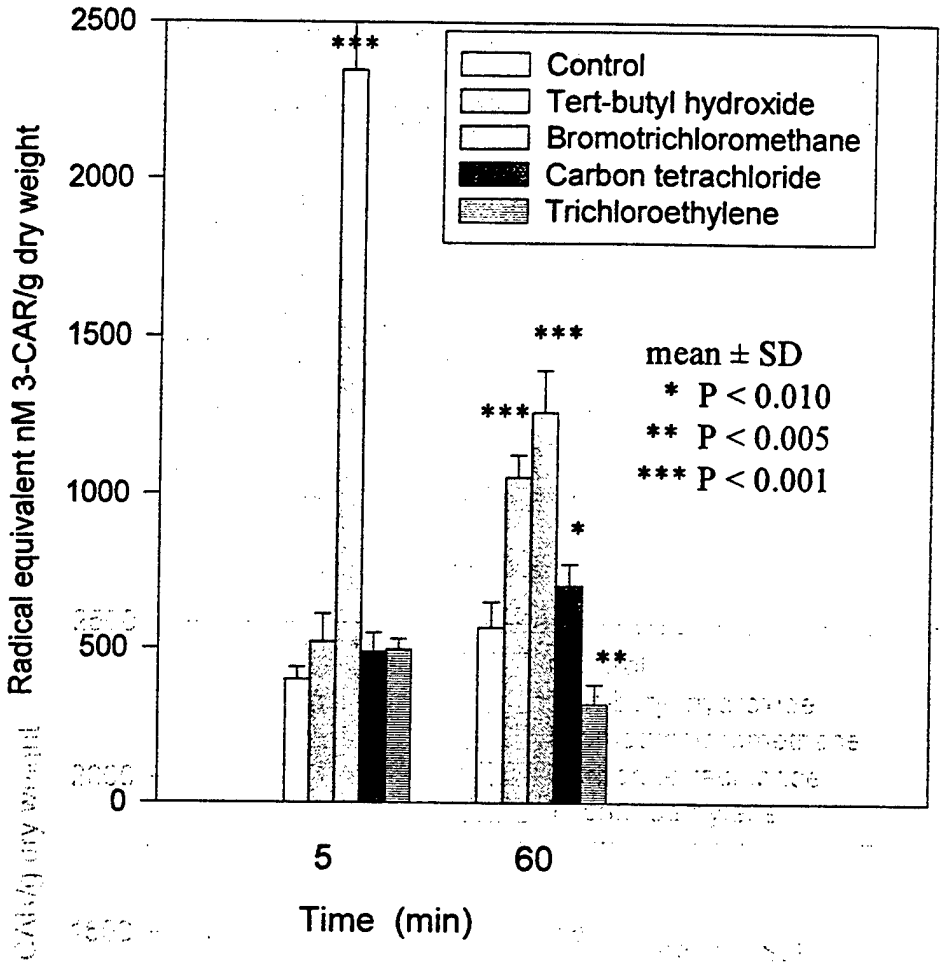

Figure 5. ***Radicals quantitated at 5 and 60 minutes in control liver slices and liver slices exposed to 1 mM TBOOH, $BrCCl_3$, CCl_4, or TCE***

Figure 5 shows the total radicals quantitated at 5 and 60 min. intervals between each group. There was a significant increase in radicals detected after 5 min. incubation with $BrCCl_3$, ($P < 0.001$) compared with controls but no difference with the other samples. However, all the treated samples were different from the control radical value at 60 min.,

($P < 0.04$). Interestingly the total radicals detected for TCE was less than the control at 60 min., $P < 0.005$, while the other chemicals showed increased numbers. When the effect of time was compared with each chemical we found there were significant differences in radicals detected in each treatment group ($P<0.002$) but again the radicals detected did not always increase with time. In these experiments we found more radicals in the control, TBOOH, and CCl_4,-treated slices at 60 min. than at 5 min. ($P<0.02$) but less radicals in the $BrCCl_3$ and TCE-treated slices. Future studies designed to quantitate free radicals induced by chemical exposure in liver slices must address the trap efficiency of PBN for the specific radical under study, the interaction of both time and concentration and especially the inter-experimental variation of media preparation. This should be done before data generated by liver slices for lipid peroxidation studies can be used as a quantitative tool.

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